Hypertension

Nox1 Overexpression Potentiates Angiotensin II–Induced Hypertension and Vascular Smooth Muscle Hypertrophy in Transgenic Mice

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Background—Reactive oxygen species (ROS) have been implicated in the development of cardiovascular pathologies. NAD(P)H oxidases (Noxes) are major sources of reactive oxygen species in the vessel wall, but the importance of individual Nox homologues in specific layers of the vascular wall is unclear. Nox1 upregulation has been implicated in cardiovascular pathologies such as hypertension and restenosis.

Methods and Results—To investigate the pathological role of Nox1 upregulation in vascular smooth muscle, transgenic mice overexpressing Nox1 in smooth muscle cells (TgSMCnox1) were created, and the impact of Nox1 upregulation on the medial hypertrophic response during angiotensin II (Ang II)–induced hypertension was studied. These mice have increased expression of Nox1 protein in the vasculature, which is accompanied by increased superoxide production. Infusion of Ang II (0.7 mg/kg per day) into these mice for 2 weeks led to a potentiation of superoxide production compared with similarly treated negative littermate controls. Systolic blood pressure and aortic hypertrophy were also markedly greater in TgSMCnox1 mice than in their littermate controls. To confirm that this potentiation of vascular hypertrophy and hypertension was due to increased ROS formation, additional groups of mice were coinjected with the antioxidant Tempol. Tempol decreased the level of Ang II–induced aortic superoxide production and partially reversed the hypertrophic and hypertensive responses in these animals.

Conclusions—These data indicate that smooth muscle–specific Nox1 overexpression augments the oxidative, pressor, and hypertrophic responses to Ang II, supporting the concept that medial Nox1 participates in the development of cardiovascular pathologies. (Circulation. 2005;112:2668-2676.)

Key Words: angiotensin • hypertension • hypertrophy • muscle, smooth • free radicals

Received January 31, 2005; revision received April 26, 2005; accepted May 10, 2005.

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The online-only Data Supplement, which contains additional information about Methods, can be found at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.538934/DC1.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.538934

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superoxide elevated after Ang II infusion,5,11–15 but treatment with antioxidants decreases Ang II–induced hypertension in rats and mice.16–19 Genetic overexpression of superoxide dismutase (SOD) also attenuates the rise in blood pressure stimulated by Ang II.8 One of the hallmarks of hypertension due to activation of the renin-angiotensin system is medial hypertrophy of large vessels.20 In vitro, Ang II–induced hypertrophy of large vessels.20 In vitro, Ang II–induced hypertrophy of large vessels.20 In vitro, Ang II–induced pressor and hypertrophic response during Ang II–induced hypertension is impaired in gp91phox-deficient mice, but gp91phox is expressed only in the endothelium and adventitia and not in the aortic media.11 This raises the question of whether medial NAD(P)H oxidases regulate hypertrophy in vivo.

On the basis of these considerations, transgenic mice overexpressing Nox1 in smooth muscle cells (SMCs) were created and used to investigate the impact of Nox1 upregulation, as occurs during hypertension and restenosis, on the pressor and medial hypertrophic response during Ang II–induced hypertension. We hypothesized that increased Nox1-derived ROS would exacerbate the increased wall thickness that occurs during hypertension and thus may also lead to elevations in blood pressure. We found that upregulation of Nox1 does indeed potentiate both the pressor and hypertrophic responses to Ang II in a ROS-dependent manner, supporting the concept that medial Nox1 participates in the development of cardiovascular pathologies.

Methods
Extended methods are provided in the online-only Data Supplement.
online-only Data Supplement. Primer sequences are indicated in Table 1.

The presence of these transgenes in the breeding pairs was confirmed by Southern blot analysis in both transgenic lines (TgSMCnox1 and TgSMCnox2) (Figure 1C). Transgenic mice heterozygous for Nox1 overexpression in SMCs and their wild-type (WT) littermates were used in experiments. All mice used in this study were between 6 and 7 months of age. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

**Treatment Groups**

Male TgSMCnox1 and WT mice were divided into 4 groups: control (saline infusion), Ang II, Tempol, and Tempol–Ang II. The mice were anesthetized with an intraperitoneal injection of 375 mg/kg 2,2,2-tribromoethanol, and micro-osmotic pumps were implanted subcutaneously in the midscapular region. Pumps delivered either saline infusion, Ang II, Tempol, or Tempol dissolved in saline was administered in separate 0.9% saline or Ang II at a rate of 0.7 mg/kg per day. For the Tempol groups, Tempol was dissolved in saline and administered in separate micro-osmotic pumps at a rate of 50 mg/kg per day. After 14 days, the animals were killed by CO2 inhalation, and their aortas were harvested for study.

**Cell Culture**

VSMCs were isolated from aortas of male TgSMCnox1 and WT mice by explant method with modifications.26 Cells were grown in DMEM containing 10% FBS, passaged by trypsinization, and used for experiments at passage 3.

**Western Blotting**

Aortas were harvested and cleaned of fat and connective tissue. Proteins from mouse aortas or cultured aortic SMCs from TgSMCnox1 and WT mice were extracted and analyzed by Western blotting as described previously.7 After incubation with horseradish peroxidase–conjugated secondary antibody, proteins were detected by ECL chemiluminescence.

**Immunofluorescent Histochemistry**

Single-label fluorescent immunohistochemistry was performed on frozen 5-μm OCT-embedded tissue sections as described previously5 with the use of a rabbit polyclonal anti-nox1 antibody23 at 1:100 dilution. Serial sections were treated with secondary antibodies alone to control for nonspecific staining.

**Real-Time Quantitative Reverse Transcriptase–PCR**

Total RNA was purified from the indicated TgSMCnox1 and WT tissues with the use of proteinase K and DNase I digestions and the RNeasy kit (Qiagen). RNA from tissue and heterologous internal luciferase standards were reverse transcribed with Superscript II enzyme (Invitrogen) with random primers. Message expression was quantified with the use of the Lightcycler instrument (Roche) with SYBR green dye and specific human or mouse nox1 or mouse gp91phox, nox4, or p22phox primers and normalized to luciferase and 18S rRNA.

**Detection of Intracellular Superoxide With High-Performance Liquid Chromatography**

To evaluate intracellular production of superoxide, we measured the formation of oxyethidium from DHE using high-performance liquid chromatography (HPLC) analysis as recently reported.27 For each experiment, three 2-mm aortic rings were incubated with 50 μmol/L dihydroethidium in fresh Krebs/HEPES buffer and homogenized in 300 μL methanol. Separation of ethidium, oxyethidium, and dihydroethidium was performed with the use of an acetonitrile gradient and a C-18 reverse-phase column (Nucleosil 250-4.5 mm) on a Beckman HPLC System. Oxidized ethidium was expressed per milligram protein. In some samples, polyethylene glycol (PEG)–SOD (100 U/mL) was added 1 hour before addition of dihydroethidium. PEG-SOD inhibited the dihydroethidium signal by 60%.

**Superoxide Detection by Electron Spin Resonance**

Aortas harvested as described above were cut into 2-mm rings, and 3 of each were incubated for 40 minutes at 37°C in 1 mL of Krebs/HEPES buffer (pH=7.4) containing 5 μmol/L DETC, 50 mmol/L Desferal, and 0.5 mmol/L CMH. Rings were then frozen in liquid nitrogen, and electron spin resonance (ESR) spectra were recorded with a Bruker EMX ESR spectrometer and a super-high-Q microwave cavity. The ESR instrument settings were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 5242.88 ms; 512-point resolution and receiver gain, 1×106. The amplitude of the signal was measured, and the concentration of CM-radical was determined by calibration with standard concentrations of CM-nitroxide. The portion of the signal due to superoxide was determined by preincubation of duplicate samples with PEG-SOD (100 U/mL) for 3 hours in Krebs/HEPES buffer. PEG-SOD pretreatment inhibited 65% to 75% of CM-nitroxide formation. The formation of CM-nitroxide was normalized to the dry weight of aorta rings.

**Systolic Blood Pressure Measurement**

Systolic blood pressure was measured with the use of tail-cuff plethysmography (Visitech Systems Inc). Blood pressure was measured twice before the implantation of osmotic pumps and on days 3, 7, 10, and 14 after pump placement. A set of 10 to 20 measurements was obtained for each animal, and the mean blood pressure was calculated. This noninvasive method of measuring blood pressure correlates well with intra-arterial measurements in normotensive and hypertensive mice.28

**Assessment of Hypertrophy**

After euthanasia, the heart and aorta were pressure-perfused at 100 mm Hg with 0.9% sodium chloride solution, followed by pressure fixation with a 10% formalin solution. Aortas were embedded in paraffin, and three 5-μm cross sections were cut starting 6 mm from the aortic arch and stained with hematoxylin and eosin. Digital

**TABLE 1. Genotyping Primers**

<table>
<thead>
<tr>
<th>Primer for</th>
<th>Primer Sequence 5’ to 3’</th>
<th>Expected Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional PCR</td>
<td>Nox1 Forward: GTGAGGATTTCCAGTATGAAG</td>
<td>308</td>
</tr>
<tr>
<td>Cre</td>
<td>Reverse: TGTCAGAATTGCTGATC</td>
<td>357</td>
</tr>
<tr>
<td>Real-time quantitative PCR</td>
<td>Nox1 Forward: TTCCACAAATCCAGGATTGAGTGGATGTC</td>
<td>379</td>
</tr>
<tr>
<td>Cre</td>
<td>Reverse: GACCTGTCAGATGGAGTGGCCTTGTG</td>
<td>123</td>
</tr>
</tbody>
</table>

**Note:** Primer sequences are indicated in Table 1.
images were obtained with the use of a Zeiss Axioskop. To quantify wall thickness, radial lines were drawn to determine the distance from internal elastic lamina to the external lamina at a minimum of 10 locations per aortic section, and a mean value was calculated. To determine cross-sectional wall area (CSWA), the perimeters of the internal and external elastic laminas were traced. The area inside each respective perimeter was determined, and the difference between these areas was reported as the CSWA. All measurements were completed with the use of NIH Image (version 1.62).

Statistical Analysis
Data are shown as mean±SE. Statistical significance was assessed by ANOVA on untransformed data, followed by comparison of group averages by contrast analysis, with the use of the Super-

ANOVA statistical program (Abacus Concepts). A probability value <0.05 was considered statistically significant.

Results
Characterization of Tg\textsuperscript{SMCnox1} Mice
As shown in Figure 1, successful creation of Tg\textsuperscript{SMCnox1} mice was confirmed by genetic methods. Overexpression of Nox1 protein in vascular smooth muscle (VSM) was confirmed by Western analysis of aortas (Figure 2A). Two specific bands (≈55 and 75 kDa) were detected in Tg\textsuperscript{SMCnox1} mice, consistent with our previous measurements of Nox1 protein.\textsuperscript{4,7} Both bands were increased upon Ang II infusion. Smooth muscle–specific
expression of Nox1 was markedly enhanced, as determined by Western analysis of cells cultured from TgSMCnox1 mouse aortas (data not shown) and immunofluorescence histochemistry (Figure 2B). Nox1 was detected in the fatty tissue surrounding the outer adventitia in both WT and TgSMCnox1 mice, indicating that Nox1 is endogenously expressed in periadventitial fat (Figure 2B).

Human nox1 expression was increased in other smooth muscle–containing tissues such as colon and heart (containing coronaries) but not in brain, liver, or spleen (Table 2), confirming specific targeting of gene overexpression. Expression of this gene was accompanied by a statistically insignificant upregulation of gp91phox mRNA but had no effect on endogenous nox1, nox4, or p22phox mRNA (Table 3). Neither gp91phox nor Nox4 protein levels were altered in TgSMCnox1 mouse aortas (Figure 2C). Catalase and manganese SOD were upregulated in these animals, whereas Cu-Zn SOD was unchanged, and extracellular SOD was decreased (Figure 3). There was no obvious effect of smooth muscle–specific overexpression of the nox1 gene on body weight (WT, 29.6 ± 0.8 g; TgSMCnox1, 28.5 ± 0.8 g; n = 20), vascular development, or other physical descriptors.

**Superoxide Production in TgSMCnox1 Mice**

To test the activity of the overexpressed Nox1 protein, we used 2 methods to measure its product, superoxide. Dihydroethidium–HPLC specifically detects intracellular superoxide, whereas CMH–ESR quantitatively detects both intracellular and extracellular superoxide. Basal superoxide levels were modestly elevated in aortas from TgSMCnox1 compared with WT aortas, especially intracellular levels, which were significantly different from control (P < 0.05) (Figure 4). Infusion of an activator of Nox1, Ang II, increased superoxide production in both transgenic and WT mice; however, this increase was dramatically enhanced in both lines of TgSMCnox1 compared with WT mice (Figure 4). The elevation of superoxide production in response to Ang II was nearly completely abolished in transgenic mice by coadministration of the antioxidant Tempol (P < 0.05). Antioxidant treatment with Tempol alone did not affect baseline ROS production in either WT or TgSMCnox1 mice.

**Effect of Nox 1 Overexpression on Blood Pressure**

Previous work has shown that the increase of blood pressure in response to Ang II infusion in mice is partially mediated by

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**TABLE 2. Expression of Human nox1 in Different Tissues of Transgenic Mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT</th>
<th>TgSMCnox1</th>
<th>Tg2SMCnox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Not detected</td>
<td>750 ± 70</td>
<td>250 ± 30</td>
</tr>
<tr>
<td>Colon</td>
<td>Not detected</td>
<td>12 ± 2</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Heart</td>
<td>Not detected</td>
<td>25 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Liver</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Brain</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Spleen</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Copy numbers are mean ± SE per 10⁶ copies of 18S cDNA.
NAD(P)H oxidase-derived ROS. We measured the effect of smooth muscle–specific Nox1 overexpression on systolic blood pressure using tail-cuff plethysmography. Basal blood pressure was unchanged in TgSMCnox1 mice compared with that of their WT littermates (Figure 5). As expected, Ang II infusion significantly increased blood pressure in both TgSMCnox1 and WT mice. Similar to the effect of Nox1 overexpression on superoxide production, the blood pressure response was significantly enhanced in Tg1 SMCnox1 mice compared with WT mice (day 14, 176±4 mm Hg in Tg1 SMCnox1 versus 154±3 mm Hg in WT). Similar results were found with the second line of transgenic mice (data not shown).

To determine whether the enhanced blood pressure response in Nox1-overexpressing mice is in fact due to the accompanying increase in superoxide production, Tempol treatment of each experimental group was performed. Coinfusion of Tempol with Ang II significantly reduced blood pressure in both Tg1 SMCnox1 and WT mice, but Tempol had no effect on basal blood pressure in either group. This reduction was more pronounced in transgenic mice. These observations suggest that Nox1-derived ROS can also influence microvascular tone.

Effect of Nox 1 Overexpression on Ang II–Induced Vascular Hypertrophy

One consequence of, or perhaps contributing factor to, increased blood pressure is hypertrophy of large vessels. Although the ROS sensitivity of this response has been established in vitro,21,22 the role of Nox1 in hypertrophy has not been studied in vivo. For this reason, we assessed aortic medial thickness and CSWA of aortas from TgSMCnox1 mice were slightly but significantly greater than those of WT mice. Ang II infusion significantly increased aortic medial thickness and CSWA in control mice and in both lines of TgSMCnox1 mice. However, both Tg1 SMCnox1 and Tg2 SMCnox1 mice developed a significantly greater hypertrophic response to Ang II compared with that in WT mice. Cotreatment of mice with Tempol attenuated Ang II–induced hypertrophy in all mice, confirming that the observed effects were associated with enhanced ROS production.

Discussion

In this study we used transgenic technology to examine the effect of upregulating a single NAD(P)H oxidase (Nox1) in a single layer of the vessel wall (media) on responses associated with hypertension. We found that upregulation of Nox1 alone does not alter the basal vascular phenotype of these animals, but that on activation of this newly expressed enzyme with Ang II, VSM Nox1 exacerbates the hypertensive and hypertrophic responses of the vasculature. These results suggest that smooth muscle–derived ROS can have a profound effect on the development of hypertensive vascular disease.

Both in vitro and in vivo studies have demonstrated a role for NAD(P)H oxidase–derived ROS in the abnormal cellular responses leading to hypertensive vascular disease (for review, see Touyz). With the relatively recent discovery of multiple gp91phox homologues, a new layer of complexity has been added to the existing questions about how these enzymes modulate vascular function. Ang II infusion upregulates multiple Nox family members (Nox1, Nox2, and Nox4), perhaps in different layers of the vessel wall,5,23,33 and although NAD(P)H oxidase activity has been shown to contribute to hypertension, it is unclear which Nox enzymes mediate this effect. Landmesser et al demonstrated that deletion of p47phox significantly attenuated the rise in blood pressure stimulated by Ang II. Additional studies by Li et al showed that the impairment of endothelium-dependent vaso-
dilation induced by Ang II is abolished in p47phox−/− mice. These reports provide definitive proof that a member of the NAD(P)H oxidase family contributes to hypertension. However, p47phox can regulate gp91phox, Nox1,35 and possibly other Nox family members, so that these studies do not permit determination of which NAD(P)H oxidase is involved.

In the present study we took advantage of Cre/lox technology to generate mice in which Nox1 is specifically overexpressed in VSM using a SM-MHC/Cre mouse previously developed and characterized by Regan et al.25 This permitted us not only to assess the role of Nox1 in vascular function but also to dissect smooth muscle–specific responses from those of the endothelium and adventitia. As expected, human nox1 mRNA was detected only in organs with smooth muscle–containing tissues, such as aorta and colon, but not in brain, spleen, or liver. However, there was an unexpectedly high expression of nox1 mRNA in heart. This may be due to the fact that real-time PCR can detect very low levels of message, such as those located in the coronary arteries in a whole-heart preparation. Alternatively, the heart is known to transiently express other genes that are normally restricted to smooth muscle.36,37 Indeed, Regan et al25 reported a small subpopulation of cells within the myocardium that activated the SM-MHC cre gene in their studies, although the precise lineage of these cells was not ascertained. Two different founder lines of mice that express different levels of nox1 mRNA had similar phenotypes with respect to superoxide, blood pressure, and vascular hypertrophy, with some dosage effect (Figures 4 to 6 and Table 3). The fact that mice with relatively low levels of Nox1 overexpression (TgSMCnox1), similar to those observed in hypertension, also exhibited enhanced hypertensive and hypertrophic responses to Ang II infusion mitigates concerns about nonphysiological overexpression of this gene.

The functionality of Nox1 overexpression was confirmed by measuring its product, superoxide (Figure 4). Two different methods, HPLC analysis of oxyethidium and ESR, were used to verify our findings. Interestingly, TgSMCnox1 mice had only a minimal increase in aortic basal superoxide production. Because our measurements reflect the balance between superoxide production and removal, this relatively small effect may be due to a compensatory upregulation of antioxidant enzyme expression or activity in these animals. Indeed, we observed increased expression of both catalase and MnSOD in TgSMCnox1 mice. Alternatively, because Nox1 requires regulatory subunits, increased expression alone may not increase activity; an activating stimulus such as Ang II may be necessary as well. This latter interpretation is supported by our observation that superoxide...
production in response to Ang II is enhanced in Tg\textsuperscript{SMCnox1} animals (Figure 4).

As noted above, a role for ROS and NAD(P)H oxidases in blood pressure regulation has previously been established. In vivo administration of antioxidants such as Tempol, vitamin E, or membrane-permeant SOD reduces Ang II–induced hypertension in rats.\textsuperscript{16,17,38} Furthermore, genetic deletion of p47phox or administration of the specific NAD(P)H oxidase inhibitor gp91-dstat partially prevents Ang II–induced increases in blood pressure.\textsuperscript{12,13} It is noteworthy that none of these interventions affect basal blood pressure, in agreement with our findings in Tg\textsuperscript{SMCnox1} mice and in WT mice treated with Tempol (Figure 5). However, in mice lacking gp91phox the reverse is true: basal blood pressures are lower, and the Ang II–induced increase in blood pressure is unaffected.\textsuperscript{11} Taken together, these data suggest that different NAD(P)H oxidases may have distinct roles in blood pressure regulation. The gp91phox-based oxidase may provide fine control of basal blood pressure by regulating the level of bioactive NO. In contrast, Nox1, and possibly additional macrovascular medial NAD(P)H oxidases such as Nox4, may serve to enhance blood pressure responses when they are upregulated and activated, such as occurs with elevated Ang II levels.\textsuperscript{5,23} This enhancement may be due to NO inactivation as NO diffuses into VSMCs but may also in part be due to mediation of vascular remodeling.

Increased Nox1 expression was also accompanied by enhanced vascular hypertrophy in response to Ang II. Our results do not permit us to determine whether the ultimate stimulus for this enhanced response is the greater wall stress produced by the elevated pressure in the g\textsuperscript{SMCnox1} compared with the WT controls or is merely a consequence of oxidative activation by Ang II. However, exacerbated hypertrophy was reversed by Tempol, confirming that it is in fact due to increased production of ROS (Figure 6). This is not unexpected because in vitro studies have demonstrated a requirement for NAD(P)H oxidase-derived ROS in Ang II–stimulated protein synthesis.\textsuperscript{22} In cultured VSMCs, H\textsubscript{2}O\textsubscript{2}, rather than superoxide, is required for the growth response.\textsuperscript{22} We recently showed that induction of hypertrophy in Ang II–infused WT mice is not inhibited by coinfusion of ebselen, suggesting that the situation is more complicated in vivo.\textsuperscript{39} The present data indicate that superoxide may play a role in vascular hypertrophy in vivo as well, because Tempol effectively inhibited Ang II–induced hypertrophy in both WT and Tg\textsuperscript{SMCnox1} mice. Alternatively, Tempol may block growth by increasing the bioavailability of nitric oxide,\textsuperscript{40} which has been shown to inhibit VSMC proliferation.\textsuperscript{41}

Previous work has shown that production of ROS by either the endothelium or adventitia can alter medial hypertrophy.\textsuperscript{11} Each layer of the vessel wall has a different complement of NAD(P)H oxidases, which in turn are differently regulated. This permits exquisitely fine control of ROS production. The interplay between the gp91-based NAD(P)H oxidase in the adventitia and the Nox1- and Nox4-based oxidases in the media of large vessels deserves further study.

In summary, using a novel transgenic mouse that overexpresses Nox1 only in smooth muscle, we have shown that enhanced production of ROS in the medial layer of the vessel wall increases blood pressure, medial thickness, and area. Although NAD(P)H oxidases are upregulated in various vascular diseases, it has been difficult to dissect the contributions of individual cell types to vascular pathologies. Tg\textsuperscript{SMCnox1} mice represent an excellent model with which to study the effect of NAD(P)H oxidase upregulation in the media. Our data suggest that ROS produced in medial SMCs may be of vital importance to the pathogenesis of vascular diseases.

Acknowledgments

This work was supported by National Institutes of Health grants HL38206 and HL58000 to K.G.K., CA84138 to J.D.L., and HL38854 and HL57353 to G.K.O.

A.D., B.L., R.C., S.D., A.S.M., A.L., and D.S.W. performed research and analyzed data. J.D.L. designed and created Tg\textsuperscript{nox1} mice. G.C. and J.M. made Tg\textsuperscript{nox1} mice. G.K.O. provided SM-MHC-Cre mice and advice on their use. D.W. and W.R.T. assisted with the hypertension model. H.H.H.W.S. provided the Nox1 antibody. K.G.K. designed the research. All authors contributed to the preparation of the manuscript.

References

The role of reactive oxygen species (ROS) in vascular diseases such as hypertension and atherosclerosis has been controversial, in part because of the disappointing negative results of the vitamin trials. However, the failure of these trials to improve cardiovascular end points does not negate the concept that oxidative stress plays a role in disease pathogenesis. Such trials necessarily treat patients with established disease, irrespective of their oxidant status and need for treatment. Vitamins target free radicals such as superoxide but have little effect on the equally potent ROS hydrogen peroxide. Practically speaking, human subjects cannot be used to investigate the cause-effect relationships between ROS and disease pathogenesis. In contrast, transgenic animals represent an ideal model to provide insight into the role of ROS in the development of vascular disease. The present study clearly demonstrates that chronic oxidative stress produced by upregulation of the NADPH oxidase Nox1 in transgenic mice aggravates vascular hypertrophy and hypertension after a 2-week infusion of angiotensin II. These effects were reversed by the superoxide scavenger Tempol, confirming that they are due to increased ROS. The results of this study suggest that rather than attempting to scavenge oxidative products once they are formed, future therapy for oxidant-related vascular diseases should be directed toward inhibiting sources of ROS generation, such as Nox1. This approach would have the advantage of specificity and the ability to prevent ROS-mediated damage before it occurs.
Nox1 Overexpression Potentiates Angiotensin II-Induced Hypertension and Vascular Smooth Muscle Hypertrophy in Transgenic Mice


Circulation. 2005;112:2668-2676; originally published online October 17, 2005;
doi: 10.1161/CIRCULATIONAHA.105.538934
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/17/2668

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Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice


Division of Cardiology and Department of Pathology, Emory University, Atlanta, GA, Department of Pharmacology, Monash University, Victoria, Australia and Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, VA

Materials and Methods

Reagents: All cell culture media and materials were purchased from the Gibco division of Invitrogen (Carlsbad, CA). Collagenase type 2 was from Worthington (Feehold, NJ). DNeasy Tissue and RNeasy Kits were from Qiagen, Inc. (Valencia, CA). Phenylmethylsulfonyl fluoride (PMSF) was from Roche (Indianapolis, USA). Nytran N membranes were from Schleicher & Schuell (Keene, NH). 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) was purchased from Alexis Corp. (San Diego, CA). Dihydroethidium was purchased from Molecular Probes (Eugene, OR). Anti-Nox1 rabbit polyclonal antibody was prepared as described. The other rabbit polyclonal antibodies were as follows: CDK4 from Santa Cruz Biotechnology (Santa Cruz, CA), catalase from Athens Research and Technology (Athens, GA), Mn SOD and Cu/Zn SOD were from Stressgen Biotechnologies (Victoria, BC, Canada) and the ec SOD antibody was described previously. The mouse gp91phox antibody (clone 54.1) was a gift from Dr. Mark Quinn. Anti-Mouse HRP-conjugated antibodies were from Amersham (Piscataway, NJ), anti-rabbit HRP-conjugatged antibodies were from Bio Rad Laboratories (Hercules, CA). All other chemicals including 2,2,2-tribromoethanol (Avertin), angiotensin II and Tempol were from Sigma-Aldrich (St. Louis, MO).

Animals

The nox1 transgene was constructed in a specially designated pBSCX1-LEL plasmid vector. The final construct consisted of the strong universal promoter designated CX1, green fluorescent protein (GFP) cDNA with a stop codon flanked by loxP DNA cis-elements, and human nox1 cDNA (Figure 1A). CX1 is a hybrid promoter consisting of a portion of the β-actin and CMV enhancer sequences and was designed specifically for high-level ubiquitous expression in all mouse tissues and cell types in vivo. Two founder lines of these transgenic mice (Tg1nox1 and Tg2nox1) were generated in David Lambeth’s laboratory by the Emory University transgenic mouse core facility.

To create transgenic mice in which Nox1 is specifically overexpressed in smooth muscle, we employed a Cre/LoxP system. Mice containing the nox1 transgene were bred with transgenic mice expressing a cre transgene composed of Cre recombinase cDNA under the control of the smooth muscle cell myosin-heavy-chain-β promotor. Both strains are on a C57Bl/6.
background. In mice positive for both the nox1 transgene and the cre transgene, Cre recombinase is expressed only in smooth muscle and excises the floxed GFP cDNA, leaving nox1 cDNA under the control of the CX1 promoter. Consequently, the human nox1 transgene is expressed exclusively in smooth muscle (TgSMCnox1)(Figure 1A).

The presence of human nox1 and cre in mouse genomic DNA was detected using a conventional PCR method (Fig. 1B). Primer sequences are indicated in Table 1. The human nox1 primers do not detect the native murine gene, because of the presence of 1.8 kb of intervening intron sequences. Detection of the nox1 and cre transgenes was also performed by real-time PCR using genomic DNA from tail clips. In this qualitative assay, the presence of each transgene is detected at the end of the amplification reaction by melting curve analysis in the presence of SYBR green dye and comparison to positive and negative controls. Primer sequences are indicated in Table 1. As in the conventional PCR method, native mouse nox1 is not detected due to the presence of introns between the primers.

The presence of these transgenes in the breeding pairs was confirmed by Southern blot analysis in both transgenic lines (Tg1SMCnox1 and Tg2SMCnox1) (Figure 1C). Transgenic mice heterozygous for Nox1 overexpression in smooth muscle cells and their wild-type (WT) littermates were used in the experiments. All mice used in this study were on a C57BL/6 background, and were between 6 and 7 months of age. All procedures were approved by the Emory University Institutional Animal Care and Use Committee and were in compliance with the standards for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD.

**Southern blot analysis of genomic DNA**

Genomic DNA was extracted from tail clips and aortas using the DNeasy Tissue Kit following the manufacturer’s recommendations. DNA was then digested with Bam HI overnight, separated on a 0.8% agarose gel, transferred onto a nylon membrane, and pre-hybridized for 2-3 hours at 42°C in ULTRAhyb (Ambion, Austin, TX). Hybridization was performed overnight with 32P-labeled cDNA probes designed to detect nox1 or cre. Final washing conditions were 20 min at 55°C in 0.1X SSC and 1%SDS for nox1 and 4 x 20 min at 62°C in 0.2X SSC and 1%SDS for cre. To prepare the cre probe, the pLC cre plasmid, obtained from the local transgenic core facility, was digested with Sal I and Pst I. The excised 1032 bp insert, corresponding to the full coding region, was purified by agarose gel electrophoresis. The human nox1 probe comprises a fragment of the coding region (nucleotides 347-1726 in accession number AF127763). It was prepared from human nox1 cDNA by PCR using primers 5'-GTCAACATTCCTGTCGCCGAGCGTC-3' and 5'-CTCCCCAGGAGGTTTTCTGTTTTCA-3'.

The resulting PCR product (1.4 kb) was purified by agarose gel electrophoresis. Probes were labeled with a random priming kit (Prime it II, Stratagene) using 32P dCTP. Bands were detected by autoradiography.

**Treatment groups**

TgSMCnox1 and WT mice were divided into four groups: Control (saline infusion), Ang II, Tempol and Tempol + Ang II. The mice were anesthetized with an intraperitoneal injection of 375 mg/kg 2,2,2-tribromoethanol, and micro-osmotic pumps were implanted subcutaneously in the mid-scapular region. Pumps delivered either 0.9% saline or angiotensin II at a rate of 0.7 mg/kg/day. For the Tempol groups, Tempol dissolved in 0.9% NaCl was administered in separate micro-osmotic pumps at a rate of 50 mg/kg/day. After 14 days, the animals were killed by CO2 inhalation, and their aortas were harvested for study.
Cell culture

VSMCs were isolated from aortas of male Tg<sup>SNCox1</sup> and WT mice by the explant method with modifications. The mice were killed by CO₂ inhalation, and aortas were removed, cut longitudinally and cleaned of endothelium and adventitia in Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics. One-mm pieces of the aorta were incubated in filtered DMEM containing 1 mg/ml collagenase for two hours and placed in a culture dish. After 20 minutes, DMEM containing 20% FBS was added. DMEM containing 10% FBS was used after passage 1. Cells were passaged by trypsinization and used for experiments at passage 3.

Western blotting

Mouse aortas or cultured aortic smooth muscle cells from Tg<sup>SNCox1</sup> and WT mice were homogenized and incubated for 30 min in 1% Triton X-100 lysis buffer (50 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanoante) containing fresh protease inhibitors (10 g/mL leupeptin, 10 g/mL aprotinin and 1 mmol/L PMSF). Samples were then centrifuged at 18,000 X g for 10 minutes, and proteins in the supernatant were separated using 12% SDS-PAGE(for SOD enzymes) or 9% SDS-PAGE( for Nox proteins and catalase), transferred to nitrocellulose membranes, blocked, and incubated overnight at 4°C with the primary antibodies. After incubation with HRP-conjugated secondary antibodies, proteins were detected by ECL chemiluminescence.

Immunofluorescent histochemistry

Single-label fluorescent immunohistochemistry was performed on frozen 5-µm OCT-embedded tissue sections as described previously using a rabbit polyclonal anti-nox1 antibody at 1:100 dilution. Serial sections were treated with secondary antibodies alone to control for non-specific staining.

Real time quantitative RT-PCR

Several tissues (liver, heart, spleen, brain, colon, and aorta) were harvested from WT and Tg<sup>SNCox1</sup> mice, and total RNA was isolated using the RNeasy Kit following the manufacturer’s recommendations for purification from tissue. Preparation of the RNA included digestion with proteinase K and DNase I to eliminate possible genomic DNA contamination that could result in false positives in the PCR. Control samples were used without reverse transcriptase (RT negative). cDNA was prepared from 5 micrograms of total RNA. Exogenous luciferase RNA (1x10<sup>8</sup> copies) was added to each sample before reverse transcription to serve as a heterologous internal control. Primer sequences used to detect luciferase by real-time PCR were 5’-GCTGCTGGTGCCACCCCTATTCTCCTTT-3’ and 5’-CGCGCAACTTTTTCGCGTTGTTACTTGA-3’. To prepare luciferase RNA, the pGL3 Basic reporter plasmid (Promega) was modified by insertion of a T7 promoter at the Xba I site, just 3’ of the luciferase coding region. A fragment of the modified plasmid, including the 3’ half of luciferase and the T7 promoter was excised by digestion with Sap I, and gel purified. This fragment was used as a template for in vitro transcription with T7 polymerase. The RNA product was digested with DNase I to remove the DNA template, purified using the RNeasy kit and quantified by UV spectrophotometry. The reverse transcription reaction was performed using Superscript II enzyme (Invitrogen) and random nanomer primers as described previously. Message expression was quantified using the Lightcycler instrument (Roche) with SYBR green
dye and specific human or mouse nox1 primers and normalized to luciferase and 18S rRNA. Our human nox1 primers do not detect murine nox1 in conditions used for real-time PCR.

**Detection of intracellular superoxide using high performance liquid chromatography**
To evaluate intracellular production of superoxide, we measured the formation of oxyethidium from dihydroethidium (DHE) using high performance liquid chromatography (HPLC) analysis as recently reported. Aortas were carefully dissected and cleaned of fat and loose connective tissue. Each aorta was cut into 10-12 2-mm rings and placed in chilled Krebs/HEPES buffer, pH 7.4. For each experiment, 3 rings were incubated for 20 min at 37°C in 1 mL of Krebs/HEPES-buffer containing 50 μmol/L DHE, washed twice, and incubated for a further hour in fresh Krebs/HEPES buffer at 37°C. Aortic rings were then homogenized in 300 μL methanol. Fifty μL of homogenate were taken for protein measurements, and the remainder was filtered through a 0.22 μm syringe filter. Separation of ethidium, oxyethidium and dihydroethidium in the filtered samples was performed on a C-18 reverse phase column (Nucleosil 250-4.5 mm) using a Beckman HPLC System equipped with both UV and fluorescence detectors. The mobile phase contained 60% acetonitrile and 0.1% trifluoroacetic acid. Dihydroethidium, ethidium and oxyethidium were separated by a linear increase in acetonitrile concentration from 37% to 47% in 23 min at a flow rate of 0.5 ml/min. Fluorescence detection at 580 nm (emission) and 480 nm (excitation) was used to monitor oxyethidium production. UV absorption at 355 nM was used for detection of dihydroethidium. Oxidized ethidium was expressed per mg protein. Protein concentration was measured by Bradford assay. In some studies PEG-SOD (100 U/ml) was added one hour prior to addition of dihydroethidium. PEG-SOD inhibited the DHE signal by 60%.

**Superoxide detection by electron spin resonance (ESR)**
Aortas harvested as described above were cut into 2-mm rings, and three of each were incubated for 40 min at 37°C in 1 mL of Krebs/HEPES buffer (pH=7.4) containing 5 μM DETC, 50 mM desferal and 0.5 mM CMH. Rings were then frozen in liquid nitrogen, and ESR spectra were recorded using a Bruker EMX ESR spectrometer and a super-high Q microwave cavity. The ESR instrument settings were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 5242.88 ms; 512 points resolution and receiver gain, 1 x 104. The amplitude of the signal was measured and the concentration of CM-radical was determined by calibration with standard concentrations of CM-nitroxide. The portion of the signal due to superoxide was determined by preincubation of duplicate samples with PEG-SOD (100 U/ml) for 3 hours in Krebs/HEPES buffer. PEG-SOD pretreatment inhibited 65-75% of CM-nitroxide formation.

After the ESR measurement, rings were transferred into Eppendorf tubes, dried for 48 hours at 37°C, and their dry weight was determined. The formation of CM-nitroxide was normalized to the dry weight of aorta rings.

**Systolic blood pressure measurement**
Systolic blood pressure was measured using tail-cuff plethysmography (Visitech Systems Inc., Apex, NC). Blood pressure was measured twice before the implantation of osmotic pumps and on days 3, 7, 10, and 14 after pump placement. A set of 10-20 measurements was obtained for each animal, and the mean blood pressure was calculated. This noninvasive method of
measuring blood pressure correlates well with intraarterial measurements in normotensive and hypertensive mice.\textsuperscript{10}

**Assessment of Hypertrophy**

Following euthanasia, the heart and aorta were pressure-perfused at 100 mm Hg with 0.9% sodium chloride solution, followed by pressure fixation with a 10% formalin solution. Aortas were embedded in paraffin, three 5-\textmu m cross-sections were cut starting 6-mm from the aortic arch, and stained with hematoxylin and eosin. Digital images were obtained using a Zeiss Axioskop. To quantify wall thickness, perpendicular lines were drawn to determine the distance from internal elastic lamina to the external lamina at a minimum of 10 locations per aortic section and a mean value was calculated. All measurements were completed using NIH image (version 1.62).

**Statistics**

Data are shown as mean ± SE. Statistical significance was assessed by analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the SuperANOVA statistical program (Abacus Concepts; Berkeley, CA). A \textit{p} value <0.05 was considered to be statistically significant.

**References**
